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Pentagalloylglucose down-regulates mast cell surface FcεRI expression in vitro and in vivo

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ABSTRACT

Mast cell activation by immunoglobulin E (IgE)-mediated stimuli is a central event in the pathogenesis of allergic disorders. The present report shows that treatment with pentagalloylglucose (PGG) resulted in a down-regulation of FcεRI surface expression on mucosal-type murine bone marrow-derived mast cells (mBMMCs), which correlated with a reduction in IgE-mediated activation of mBMMCs. Furthermore, PGG prevented development of allergic diarrhea in a food-allergy mouse model and suppressed the up-regulated FcεRI surface expression on mast cells derived from the food-allergy mouse colon. These findings on PGG suggest its therapeutic potential for allergic diseases through suppressing the FcεRI surface expression.

1. Introduction

Allergic diseases are characterized by increases in the number of mast cells and the plasma level of immunoglobulin E (IgE) antibodies that signal through high-affinity IgE receptors (FcεRI) to induce the release of inflammatory mediators from mast cells. There are two distinct populations of mast cells, namely, mucosal mast cells and connective-tissue mast cells. So far, there has been considerable evidence demonstrating that mucosal mast cells are morphologically, biochemically, and functionally distinct from connective-tissue mast cells [1–4].

We found that the number of mucosal mast cells was greatly increased in the colons of the food-allergy model mice [5]. It is now clear that the mast cell's functional responses to IgE-dependent stimulation can be greatly influenced by the level of expression of FcεRI on the cell surface. It was reported that IgE can potently enhance the FcεRI surface expression of mast cells and show enhanced sensitivity and a stronger response to antigenic stimulation [6]. On the other hand, interleukin (IL)-4, IL-10, and transforming growth factor-β1 (TGF-β1) have been shown to down-regulate FcεRI expression and thereby inhibit IgE-mediated cytokine production in mast cells [7].

Previously, we found that Kakkonto, a traditional Japanese herbal medicine, significantly suppressed the occurrence of allergic diarrhea in the food-allergy model mice [5]. Because mucosal mast cells play an important role in the development of food-allergy symptoms [8], it was hypothesized that Kakkonto may have a direct effect on mucosal mast cells. The present study investigated the effect of Kakkonto and its components on mBMMCs. Our results demonstrate that 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (pentagalloylglucose, PGG), a constituent of Kakkonto, suppressed mast cells function through down-regulating the FcεRI surface expression both in vitro and in vivo.

2. Materials and methods

2.1. Cell culture and degranulation assay

mBMMCs were prepared from BALB/c mice according to the method described previously [9,10]. Rat basophilic leukemia (RBL)-1 cells were provided by Health Science Research Resources

Abbreviations: BSA, bovine serum albumin; DNP, dinitrophenyl; FcεRI, high-affinity IgE receptors; GA, gallic acid; IgE, immunoglobulin E; IL, interleukin; LPMC, lamina propria mononuclear cell; mBMMC, mucosal-type murine bone marrow-derived mast cell; mMCP-1, mouse mast cell protease-1; OVA, ovalbumin; PGG, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose; TGF-β1, transforming growth factor-β1; TGG, 1,3,6-tri-*O*-galloyl-b-D-glucose

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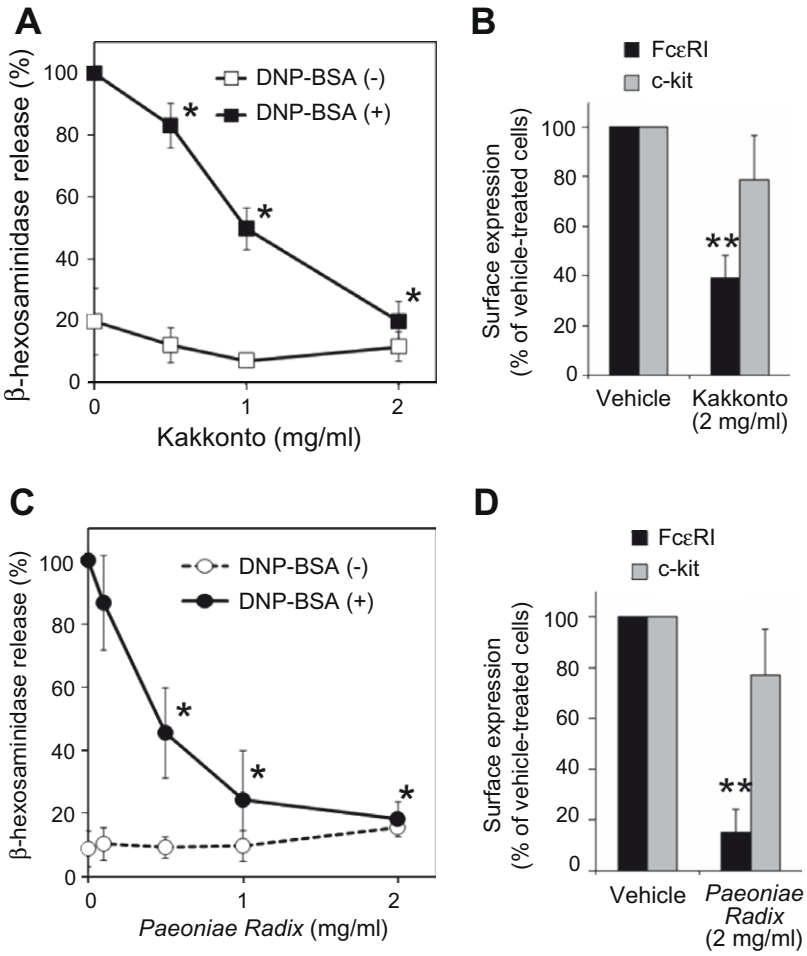

Fig. 1. Kakkonto and *Paeoniae Radix* inhibit mBMMC degranulation and down-regulates surface expression of high-affinity IgE receptors (FcεRI). (A) mBMMCs were sensitized with immunoglobulin E (IgE) anti-dinitrophenyl (DNP), and activated by DNP-bovine serum albumin (BSA). Kakkonto at the concentrations noted was added during pre-incubation (24 h) and sensitization (6 h), stimulated with DNP-BSA for 1 h, and β-hexosaminidase release was determined. Data represent mean ± S.D. values of three experiments. $^{*}P < 0.05$ compared to vehicle-treated cells. (B) mBMMCs were cultured with Kakkonto (2 mg/mL) for 24 h, and surface expression levels of FcεRIα (filled) and c-kit (gray) were determined by flow cytometry. Data represent mean ± S.D. values of four experiments. $^{**}P < 0.01$ compared to vehicle-treated cells. (C) *Paeoniae Radix* extracts at the concentrations noted were added during pre-incubation and sensitization. The treated cells were stimulated with (filled) or without (open) DNP-BSA for 1 h, and β-hexosaminidase release was determined. Data represent mean ± S.D. values of three experiments. $^{*}P < 0.05$ compared with vehicle-treated cells. (D) mBMMCs were cultured with *Paeoniae Radix* (2 mg/mL) extracts for 24 h, and the surface expression levels of FcεRIα (filled) and c-kit (gray) were determined by flow cytometry. Data represent mean ± S.D. values of three experiments. $^{**}P < 0.01$ compared to vehicle-treated cells.

Table 1
Medicinal plant compositions of various Japanese herbal medicines.

	Kakkonto (%)	Keishito (%)	Syomakakkonto (%)	Syakuyakukanzoto (%)	Hochuekkito (%)	Shikunshito (%)
Paeoniae Radix	11	26	25	50		
Glycyrrhizae Radix	11	13	13	50	6	7
Puerariae Radix	22		42			
Zizyphi Fructus	17	26			8	7
Ephedrae Herba	17					
Cinnamomi Cortex	11	26				
Zingiberis Rhizoma	11	10	4		2	7
Cimicifugae Rhizoma			17		4	
Bupleuri Radix					8	
Atractylodis Lanceae Rhizoma					17	27
Ginseng Radix					17	27
Poria						27
Astragali Radix					17	
Angelicae Radix					13	
Aurantii Nobilis					8	
β-Hexosaminidase release (%)	15.7 ±4.9**	14.1 ±4.4**	15.0 ± 8.7**	24.2 ± 17.4**	68.8 ± 8.7	66.7 ± 10.4

Data of β-hexosaminidase release represent mean ± SD values of three experiments.
** $P < 0.01$ compared with vehicle-treated cells.

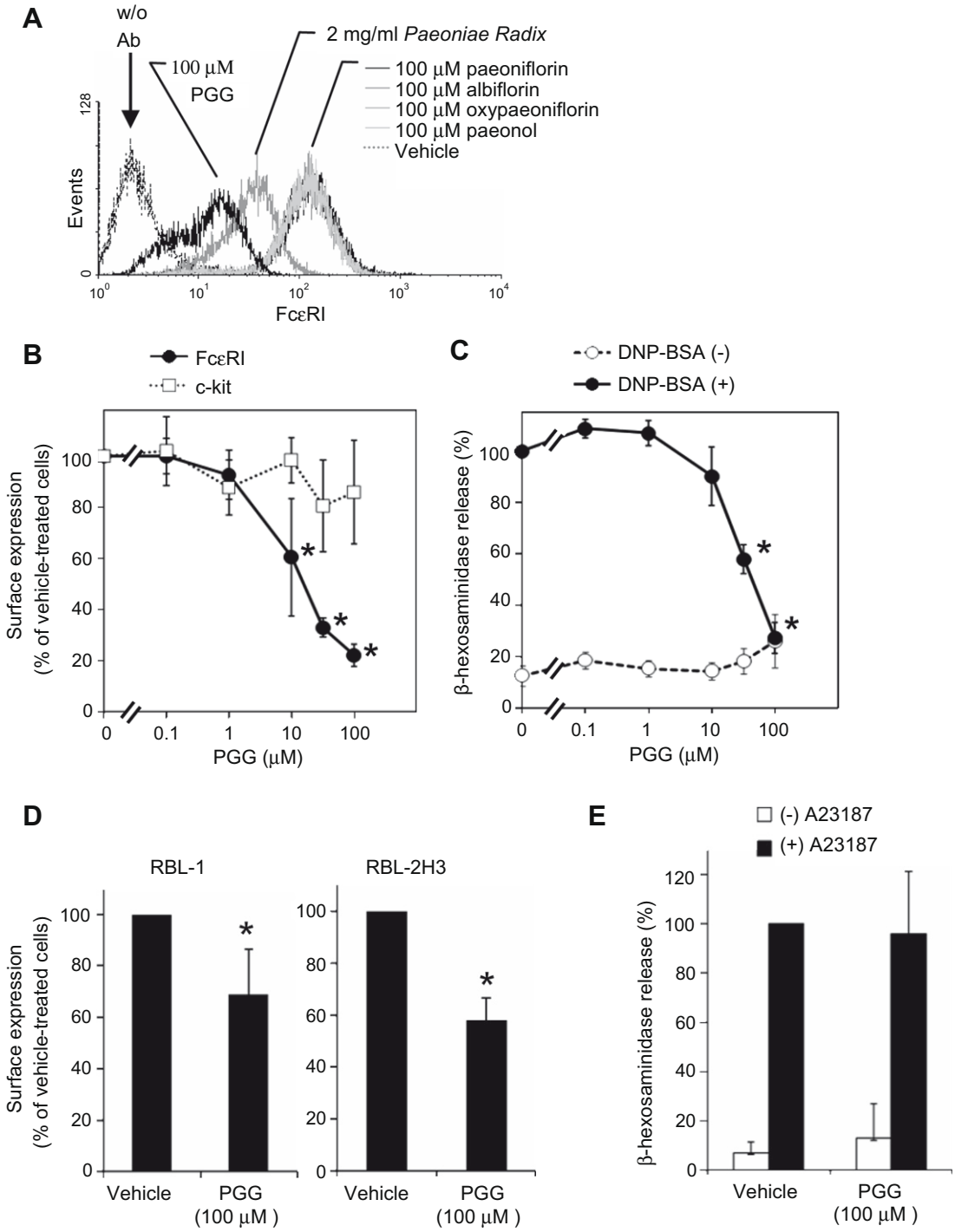

Fig. 2. 1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose (PGG) inhibits mBMMC degranulation and down-regulates surface expression of FcεRI. (A) mBMMCs were incubated with 100 μM of PGG, paeoniflorin, albiflorin, oxypaeoniflorin, paeonol and vehicle for 24 h, and the surface expression levels of FcεRI were determined by flow cytometry. (B) mBMMCs were incubated with PGG at the concentrations noted for 24 h, and the surface expression levels of FcεRI and c-kit were determined by flow cytometry. Data represent mean ± S.D. values of 4 experiments. $^{*}P < 0.05$ compared with vehicle-treated cells. (C) PGG at the concentrations noted was added during pre-incubation (24 h) and sensitization (6 h). The treated cells were stimulated with (filled) or without (open) DNP-BSA for 1 h, and β-hexosaminidase release was determined. Data represent mean ± S.D. values of four experiments. $^{*}P < 0.05$ compared with vehicle-treated cells. (D) RBL-1 and RBL-2H3 cells were treated with 100 μM PGG and vehicle for 24 h and surface expression levels of FcεRI were determined by flow cytometry. Data represent mean ± S.D. values of four experiments. $^{*}P < 0.05$ compared with vehicle-treated cells. (E) mBMMCs were incubated with 100 μM PGG or vehicle for 24 h, and the cells were stimulated with (filled) or without (open) 1 μM calcium ionophore A23187 for 20 min. Release of β-hexosaminidase was determined. Data represent mean ± S.D. values of three experiments.

Bank (JCRB No. IFO50034; Osaka, Japan) and RBL-2H3 cells were kindly provided by Dr. Hidetaka Yakura (Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan).

The degree of degranulation was assessed by measuring β-hexosaminidase release as previously described [10].

2.2. Flow cytometry analysis

mBMMCs were washed with FACS buffer (PBS, 1% bovine serum albumin (BSA), 0.2% NaN_3), stained with FITC-labeled anti-mouse FcεRI alpha (clone MAR-1, eBioscience, San Diego, CA) and

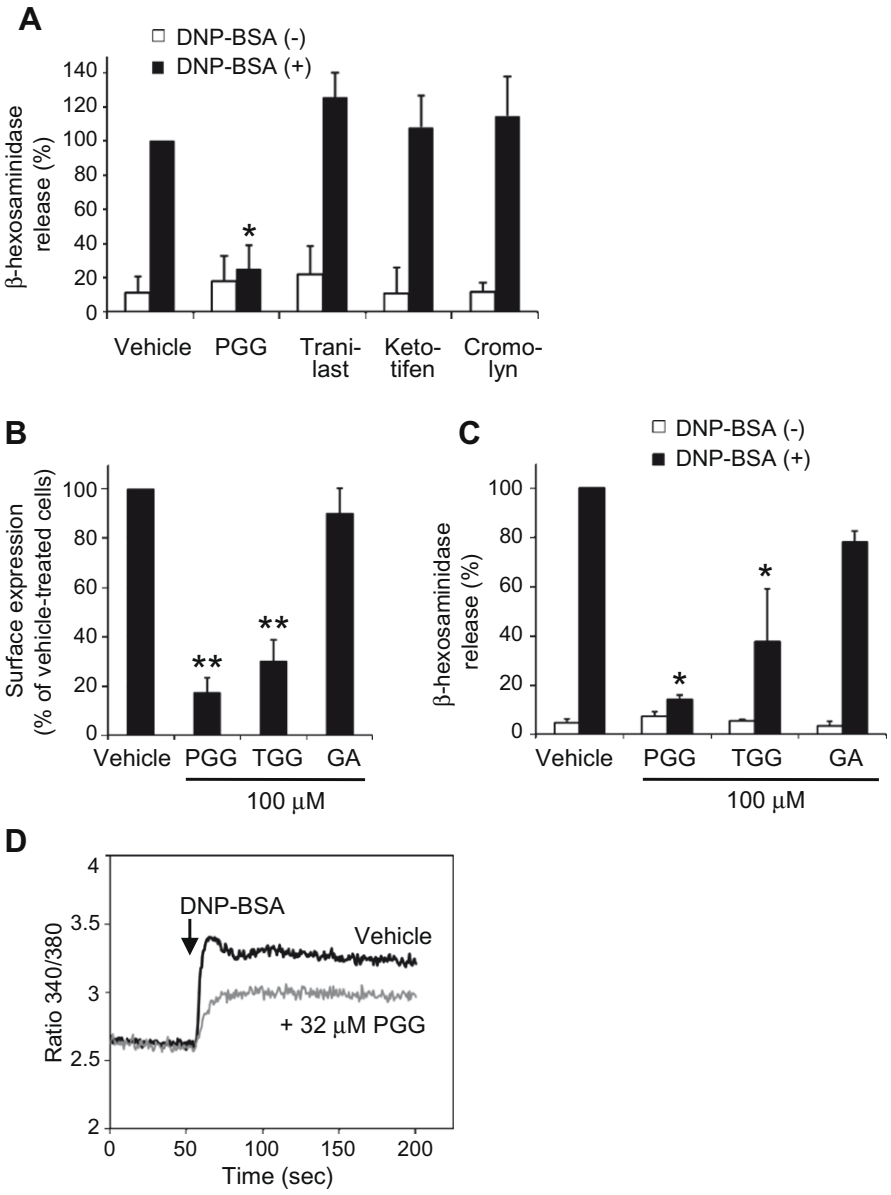

Fig. 3. (A) Tranilast, ketotifen, and cromolyn (10 μM each) were added to mBMMCs 30 min before being activated by the antigen DNP-BSA, and the extent of degranulation was assessed. The cells treated with vehicle or 100 μM PGG during pre-incubation (24 h) and sensitization (6 h) were used as controls. Data represent mean ± S.D. values of three experiments. $^{*}P < 0.05$ compared with vehicle-treated cells. (B and C) Effects of 1,3,6-tri-*O*-galloyl-b-D-glucose (TGG) and gallic acid (GA) on the surface expression levels of FcεRI (B) and degranulation (C). mBMMCs were treated with 100 μM PGG, TGG, GA and vehicle for 24 h, and surface expressions of FcεRI were determined by flow cytometry (B). After pre-incubation and sensitization, the treated cells were stimulated with (filled) or without (open) DNP-BSA for 1 h, and β-hexosaminidase release was determined (C). Data represent mean ± S.D. values of three experiments. $^{*}P < 0.05$ compared with vehicle-treated cells. (D) Ca^{2+}-mobilization profiles, monitored by Fura-2 AM. mBMMCs were treated with 32 μM PGG or vehicle (control), sensitized with IgE, and labeled with Fura-2 AM. Ca^{2+}-mobilization was determined after stimulation with DNP-BSA. Results shown are representative of four separate experiments.

PE-labeled anti-mouse CD117 (c-kit) (clone ACK45, BD Pharmingen, San Diego, CA) for 30 min at 4 °C. RBL-1 and RBL-2H3 cells were stained with mouse anti-rat FcεRI antibody (clone BC4, BD Pharmingen), followed by FITC-labeled anti-mouse IgG. For lamina propria mononuclear cells (LPMCs), cells were incubated with FcR blocking reagent mouse (MACS, Auburn, CA) for 5 min and stained with FITC-labeled anti-mouse IgE and PE-labeled anti-mouse CD117. Propidium Iodide (Sigma) and Viaprobe (BD Pharmingen) were used to discriminate between dead and live cells. Flow cytometric analysis was performed using FACSCalibur and Cell QuestPro software version 6.0 (BD Biosciences, Franklin Lakes, NJ). Percent inhibition of FcεRI expression was determined using mean fluorescence intensity (MFI), with vehicle-treated cells as the baseline for all comparisons.

2.3. Intracellular calcium measurements

Sensitized mBMMCs with mouse anti-dinitrophenyl (DNP) IgE antibodies were loaded with 5 μM Fura-2 AM (Dojindo, Kumamoto, Japan) in loading buffer (118 mM NaCl, 4.7 mM KCl, 1 mM Na_2HPO_4, 1.13 mM $MgCl_2$, 10 mM HEPES, 5.5 mM D-glucose, 100 mM L-glutamine, 1.3 mM $CaCl_2$, 2% MEM non-essential amino acids, 0.2% BSA) for 30 min, washed once, resuspended in 1.3 mL of loading buffer and warmed to 37 °C in the cuvette. Fluorescence was measured at 340 and 380 nm using a model F-4500 fluorescence spectrophotometer intracellular Ca^{2+} measurement system (Hitachi, Tokyo, Japan), and the background-corrected 340:380 ratio was calibrated.

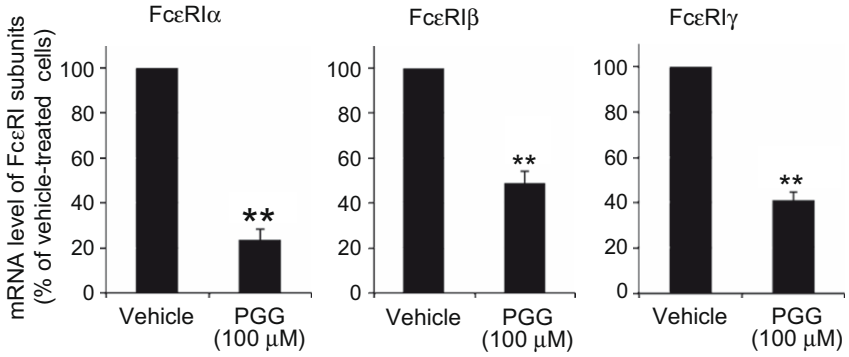

Fig. 4. PGG affects the mRNA expression level of FcεRI subunits. mBMMCs were cultured with 100 μM PGG and vehicle for 24 h and mRNA levels of α, β, and γ subunits of FcεRI were analyzed by real-time PCR as described in the material and methods. Relative mRNA levels of FcεRI subunits were normalized to GAPDH expression. Data represent mean ± S.D. values of three experiments. $^{**}P < 0.01$ compared with vehicle-treated cells.

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from mBMMCs using RNeasy Plus Micro (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was performed using PrimeScript RT reagent kit (Takara Bio, Shiga, Japan) and random primers. Quantitative real-time PCR was performed using SYBR Premix Ex Taq (Takara Bio) and Mx3000p system (Stratagene, La Jolla, California, USA). The following primer pairs were used: FcεRIα, forward 5′-TGAATGACAGTGGCACCTACCA-3′ and reverse 5′-CAGAATCGCCACCAACAATG-3′, FcεRIβ, forward 5′-AGTGCTGTGTTGTTCACTAT-3′ and reverse 5′-TCCAACTCACTGTAAATTGG-3′, FcεRIγ, forward 5′-ATATCCTGGATGCTGTCCTG-3′ and reverse 5′-TCTCATATGTCTCCTGGCTC-3′ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-TGACCACAGTCCATGCCATC-3′ and reverse 5′-GACGGACACATTGGGGGTAG-3′. Target mRNA was normalized to GAPDH mRNA as an internal control in each sample. Results were expressed as the ratio relative to the vehicle-treated cells.

2.5. Allergic diarrhea induction in a murine food-allergy model, LPMC isolation, histological analysis, and measurement of plasma histamine level

Five-week-old male BALB/c mice were housed in the experimental animal facility at University of Toyama. Intestinal anaphylaxis was induced as previously described [5]. For the treatment with PGG (Toronto Research Chemicals Inc., Ontario, Canada) in the food-allergy model, PGG was orally administered to mice everyday throughout the period of oral ovalbumin (OVA) administration. All animal care and experiments were approved by the Animal Experiment Committee in University of Toyama (Authorization No. INM-13). LPMC were isolated by the procedure described previously [11]. For histological analysis, the proximal colon was excised one hour after the oral OVA challenge, and stained with antibodies against mouse mast cell protease (mMCP)-1 by the procedure described previously [5]. The jejunum was stained with toluidine blue at pH 0.5 for 48 h. The plasma samples were obtained one hour after the oral OVA challenge, and the histamine levels were measured using ELISA (SPI Bio, Paris, France).

2.6. Statistical analysis

Statistical comparisons were made using one-way ANOVA followed by Bonferroni/Dunn post-hoc tests for multiple comparisons. In some figures, means were compared with a hypothetical value of 100 using a one-sample *t*-test. Probability values (*P*) of <0.05 were considered as statistically significant.

3. Results and discussion

3.1. Kakkonto inhibits mBMMC degranulation and surface expression of FcεRI

mBMMCs were treated with serial concentrations of Kakkonto (Tsumura Co., Tokyo, Japan) during pre-incubation (24 h) and sensitization with IgE anti-DNP (6 h), and the extent of degranulation was assessed by β-hexosaminidase release (Fig. 1A). Kakkonto (⩾0.5 mg/mL) inhibited the degranulation of mBMMCs in a dose-dependent manner.

As assessed by flow cytometry to detect FcεRI and c-kit, we found that the levels of FcεRI expression on the surface of mBMMCs were greatly inhibited upon incubation of the cells in vitro with Kakkonto (2 mg/mL). Interestingly, the surface expression levels of c-kit were not changed by treatment with Kakkonto (Fig. 1B).

3.2. Paeoniae Radix inhibits mBMMC degranulation and surface expression of FcεRI

Kakkonto is composed of seven medicinal herbs: *Puerariae Radix*, *Cinnamomi Cortex*, *Zizyphi Fructus*, *Paeoniae Radix*, *Ephedrae Herba*, *Zingiberis Rhizoma*, and *Glycyrrhizae Radix*. To determine which medicinal herb has an inhibitory effect on degranulation of mBMMCs, we used various herbal medicines (Tsumura Co.) listed in Table 1, in which the composition of medicinal herbs are similar to Kakkonto, and their effects on mBMMCs were tested. Among them, every medicine which showed the inhibitory effect on degranulation contains *Paeoniae Radix*. We then tested the effect of *Paeoniae Radix* (Tsumura Co.) on mBMMCs. As expected, it was found that treatment with *Paeoniae Radix* extract decreased the expression levels of FcεRI on the cell surface of mBMMCs, and that ⩾0.5 mg/mL of *Paeoniae Radix* significantly inhibited the degranulation of mBMMCs (Fig. 1C and D).

3.3. PGG inhibits surface expression of FcεRI and function on mBMMCs in vitro

We investigated inhibitory effects of major constituents of *Paeoniae Radix*, paeoniflorin (Nacalai Tesque, Kyoto, Japan), albiflorin (Wako Pure Chemical Industries, Osaka, Japan), oxypaeoniflorin (a gift from Tsumura Co.), paeonol (Wako), and PGG, on the FcεRI surface expression. As shown in Fig. 2A, only PGG drastically decreased the FcεRI surface expression on mBMMCs, whereas the other four components did not. The concentration-dependency of PGG on the surface expression of FcεRI and DNP-BSA-induced degranulation were tested (Fig. 2B and C). PGG at a concentration

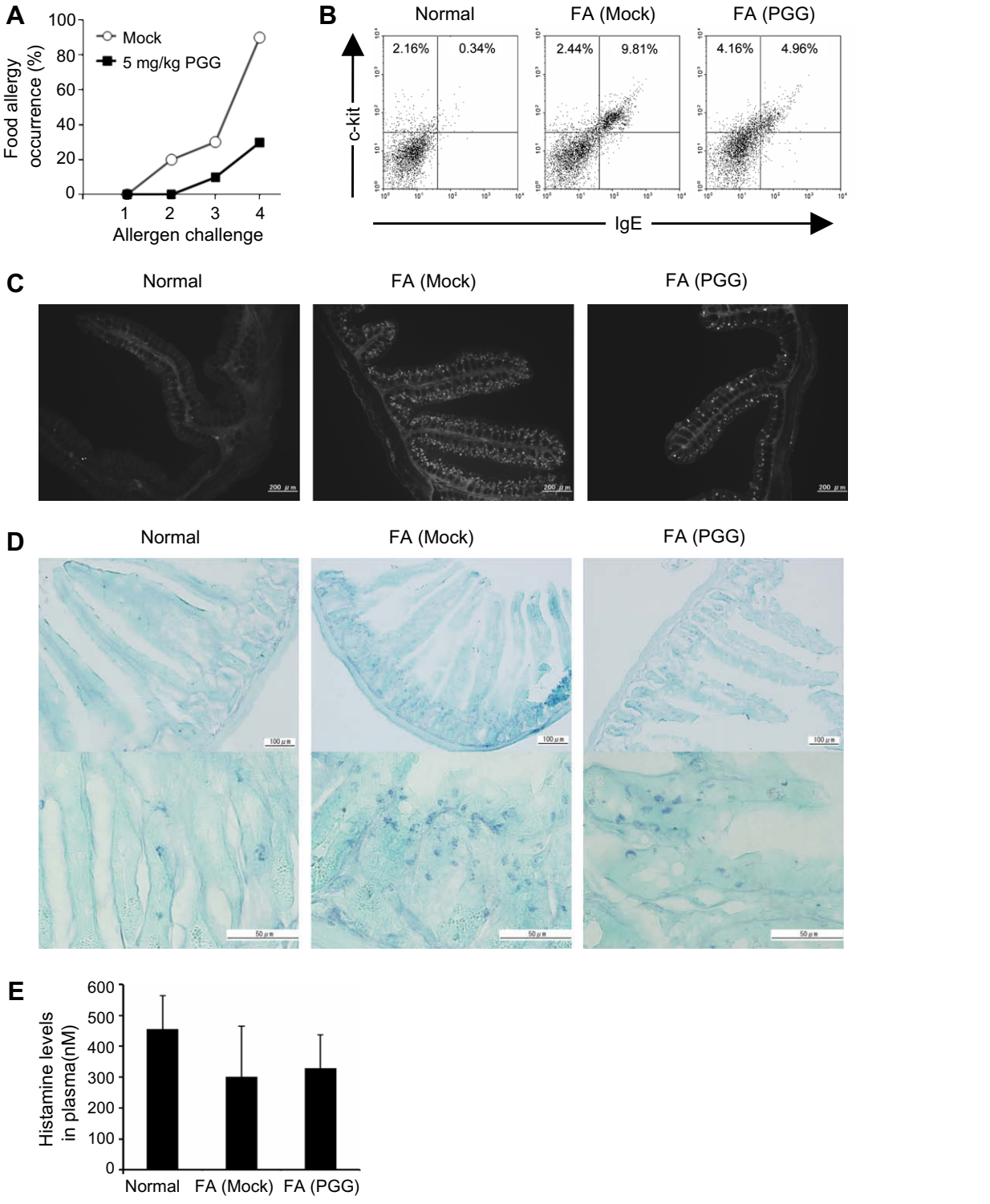

Fig. 5. PGG suppresses the development of allergic diarrhea. (A) Allergic diarrhea induction in mock- and PGG-treated groups was compared. PGG (5 mg/kg) was orally administered during induction of allergic diarrhea by OVA oral challenge as described in Material and Methods. Similar results were obtained from three independent experiments. Mock, $n = 10$; 5 mg/kg PGG, $n = 20$. (B) Mononuclear cells from large intestinal lamina propria were isolated from normal, mock-treated, and PGG-treated mice after four challenges with oral OVA (FA). The percentage of IgE-bound c-kit$^+$ mast cells was determined by flow cytometry. Results shown are representative of three separate experiments. Percentages of IgE-bound and -unbound cells in c-kit$^+$ cells are shown in each panel. (C) The proximal colon of normal, mock-treated, and PGG-treated mice after oral challenges with oral OVA (FA) was stained with anti-mouse mast cell protease-1 (mMCP-1) antibodies. Scale bars represent 200 μm. (D) The jejunum of normal, mock-treated, and PGG-treated mice was stained with toluidine blue. Scale bars represent 100 μm (upper panels) and 50 μm (lower panels). (E) The histamine levels in plasma of normal, mock-treated, and PGG-treated mice were measured using ELISA.

$\geqslant$10 μM down-regulated FcεRI expression. The down-regulation effect on FcεRI was consistently found in rat mast cell lines RBL-1 and RBL-2H3 (Fig. 2D), making it unlikely that the present data are related to culture artifacts or species differences. PGG ($\geqslant$32 μM) inhibited the degranulation of mBMMCs (Fig. 2C). In contrast, 100 μM PGG failed to affect calcium ionophore A23187

(Sigma)-induced degranulation of mBMMCs (Fig. 2E). These results suggested that FcεRI-dependent activation, but not FcεRI-independent activation of mast cells is inhibited by PGG treatment.

Interestingly, PGG down-regulated surface expression of FcεRI on mBMMCs without affecting the level of c-kit surface expression (Fig. 2B). Although IL-4, IL-10, and TGF-β1 have been shown to down-regulate FcεRI surface expression in mast cells [7], these cytokines are also known as inhibitors of c-kit expression [12,13]. c-kit has been shown to be not only essential for mast cell growth and differentiation but also to act on multipotential stem and progenitor cells, megakaryocytes, and a subset of lymphoid progenitors [14]. Therefore, PGG has the therapeutic advantage of minimizing side effects, because PGG had no influence on the surface expression of c-kit.

In the present evaluation system, none of tranilast (Sigma), ketotifen (Sigma), and cromolyn (Sigma) that inhibit the release of chemical mediators from connective-tissue mast cells in vitro [15], exerted inhibitory effects on the degranulation of mBMMCs (Fig. 3A), suggesting that PGG has a stronger inhibitory effect on mucosal mast cells than these anti-allergic drugs.

PGG contains one glucose molecule and is esterified with five gallic acids on five hydroxyl groups of the glucose. The effect of 1,3,6-tri-*O*-galloyl-b-D-glucose (TGG, ChromaDex, Irvine, CA) was lower than that of PGG, and gallic acid (GA, Nacalai Tesque) exhibited no detectable change in FcεRI surface expression and in degranulation (Fig. 3B and C), suggesting that the gallate structure of PGG, not the gallic acid moieties, may play a critical role in the down-regulation of FcεRI.

The cross-linking of FcεRI by antigen triggers mast cell activation, leading to the elevation intracellular of concentration of Ca^{2+} ($[Ca^{2+}]_i$) prior to the exocytosis of granules [16]. To examine the effect of PGG on $[Ca^{2+}]_i$ elevation in mast cells, mBMMCs were treated with 32 μM PGG for 24 h and $[Ca^{2+}]_i$ was monitored using Fura-2 AM. As shown in Fig. 3D, PGG treatment inhibited the increase of $[Ca^{2+}]_i$ triggered by DNP-BSA.

3.4. PGG inhibits mRNA expression of FcεRI subunits

FcεRI is composed of three subunits: α, β, and γ. To examine whether the mRNA expression level of FcεRI is altered by treatment with PGG, mBMMCs were treated with 100 μM PGG or vehicle for 24 h, and mRNA levels of FcεRI subunits were examined by quantitative real-time PCR. As shown in Fig. 4, PGG decreased mRNA expression of FcεRIα, β, and γ subunits. It has been reported that PGG inhibits gene expression of IL-8 in human monocytic U937 cells [17] and that of IL-1β, IL-6, and tumor necrosis factor-α in human mast cells [18]. Although the precise mechanisms remain to be determined, PGG may also be a potent inhibitor of FcεRI gene expression.

3.5. PGG down-regulates the FcεRI expression of mast cells in vivo and prevents antigen-specific allergic diarrhea

We next investigated whether PGG affected the FcεRI surface expression of mucosal mast cells in an in vivo allergic model. As shown in Fig. 5A, the induction of allergic diarrhea was partially prevented in PGG-treated mice. To examine whether PGG actually down-regulated FcεRI surface expression in mucosal mast cells in vivo, mononuclear cells were isolated from the large intestinal lamina propria and IgE-bound c-kit$^+$ mast cells were determined by flow cytometry (Fig. 5B). The number of mast cells was dramatically increased in the mock-treated food-allergy mice compared with normal mice. This result was consistent with our previous result as observed by immunohistochemical analysis [5]. PGG treatment reduced the surface-binding of IgE, suggesting that PGG down-regulated the surface expression level of FcεRI even in the in vivo food-allergy model. It has been shown that FcεRI surface expression is up-regulated by IgE both in vivo and in vitro [19,20]. In this model, however, Kakkonto treatment did not alter the level of IgE in the plasma [5], suggesting that the down-regulation of FcεRI surface expression on mast cells is not due to reduction of IgE level in the plasma.

To examine whether PGG indeed inhibited the mast cell activity in vivo, we stained the mice colon and the jejunum using antibody against mouse mast cell protease-1 (mMCP-1), a marker of mouse mucosal mast cells (Fig. 5C), and toluidine blue (Fig. 5D), respectively. Consistent with our previous report [5], the number of mast cells was dramatically increased in the intestine of the food-allergy mice compared to that of normal mice. PGG treatment greatly decreased the number of infiltrating mast cells (Fig. 5C and D), suggesting that the allergic symptoms were ameliorated by the administration of PGG. We could not see the difference in histamine levels in plasma among normal, mock-treated, and PGG-treated mice (Fig. 5E). This result is convincing because it is reported that the histamine content is much lower in mucosal mast cells than in connective-tissue mast cells [4], and that a combination of histamine H1 and H2 receptor antagonists cannot alleviate allergic diarrhea in another murine food-allergy model resembling the model we used in the present study [23].

4. Conclusion

In summary, our results demonstrate that PGG down-regulated FcεRI surface expression in mast cells both in vitro and in vivo, and thereby inhibited the degranulation of these cells in response to IgE-dependent activation. Omalizumab therapy, a clinically effective monoclonal anti-IgE capable of preventing IgE binding to FcεRI, reduced surface IgE receptor expression [21,22]. Our findings presented herein provide further support for the notion that down-regulation of FcεRI is functionally significant, as it diminishes the allergic reaction. The inhibitory effects of PGG on FcεRI surface expression may offer a new potential therapeutic approach for allergy.

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